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5 mg sVpr1-96 was dissolved in water, mixed with complete Freund's adjuvant and was used for standard immunization of rabbits. Serum samples of all bleedings were pooled, aliquoted and frozen, and the antiserum was termed "R-96". In order to establish the lowest sensitivity of R-96 for detecting Vpr, the antiserum was tested in immunoprecipitation using serial dilutions of sVpr1-96, starting from 0.1 to 10 ng. To each dilution 200 μ l of human serum derived from a healthy HIV-1 seronegative blood donor was added and the samples were further diluted by addition of 1 mL phosphate buffered saline (PBS) and then subjected to immunoprecipitation with R-96. For this purpose, to each dilution 5 μ l of R-96 adsorbed onto 30 μ l of Protein-G Sepharose beads (GammaBind-G-Sepharose-beads, Pharmacia LKB Biotechnology, Piscataway, NJ, USA) were added.

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Prior to immunoprecipitation with R-96, all dilutions were pre-cleared with Protein-G Sepharose beads loaded with rabbit IgG derived from pre-immune sera. The immune precipitation was conducted using standard conditions as described previously (Schubert and Strebel, 1994). The precipitates were denatured by boiling in SDS gel sample buffer (2% SDS, 1% β -Mercaptoethanol, 1% Glycerol, 65 mM Tris-hydrochloride (pH 6.8) (10 min, 95°C), separated in 12.5% denaturing SDS-polyacrylamide gel (SDS-PAGE, 12.5% Acryl aide gels, FMC Bioproducts, Rockland, ME) (see Fig. 2B). A dilution of the peptide sVpr1-96 (from 0.01 to 10 nM) was separated in the same gel (Fig. 2A). The separated samples were electrotransferred onto Immobilon polyvinylidene difluoride (PVDF)-Membrane (Millipore Corp., Bedford, MA), the membranes were blocked with 5% BSA in PBS (0.3% Tween 20) and finally incubated with a 1:1000 dilution of R-96 in 3% BSA. Bound antibodies were stained with ¹²⁵I-Protein G (0.1 mCi/ml; New England Nuclear, DuPont, Wilmington, DE) and visualized by autoradiography (Fig. 2). The results of this Western blot analysis demonstrate, that under those conditions 01 ng of sVpr1-96 can be detected in human serum samples using the anti serum R-96. The lowest sensitivity to detect sVpr1-96 by immune precipitation was in the range of 1 to 10 nM sVpr1-96. In other experiments using Western blot as well as immune precipitation techniques (data not shown) show that R-96 also reacts with native viral Vpr, expressed in HIV-1 infected cells.

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Example 17:

sVpr1-96 increases virus replication and number of living cells in cultured human PBMCs. For the purpose of HIV infection, parallel cultures of PBMCs isolated from peripheral blood of HIV-1-seronegative healthy blood donors were stimulated by incubation with phytohaemagglutinin (PHA) and interleukin 2 (IL-2) according to previously published methods (Schubert et al., 1995). Activated PBMCs were infected with equal infectious doses of purified virus stocks of the T cell tropic isolates HIV-1NL4-3. The culture was split and one half of the infected culture was treated with 10 nM sVpr1-96, and the other half was incubated with a 10 nM solution of the control peptide, Vpu32-81, which is biologically inert to PBMCs and was synthesized and purified under the same conditions as sVpr1-96 (Henklein et al., 1993). The control peptide Vpu32-81 comprises the 50 amino acid long cytoplasmic domain of the HIV-1 specific virus protein U (Vpu) (Wray et al., 1996). Treatment with peptides was maintained throughout the entire course of the experiment, approximately 80% of the culture medium was replaced every other day with fresh media containing the freshly prepared peptide solutions. For the purpose of estimating the amount of released virus particles, aliquots of cell culture supernatants were frozen at -80°C . At the end of the study, the activity of virus associated reverse transcriptase (RT) was estimated in parallel reactions for each cell culture supernatant sample and plotted against the time in histograms (Figure 3A). Based on those RT-profiles, summarized in Figure 3A, it can be clearly seen that, in the presence of sVpr1-96, an approximately two-fold increase in virus replication occurred during the spread of the infection in the cell culture. This sVpr1-96 induced activation of virus replication begins two days post infection and is maximal at day seven post infection, the peak of virus replication, after then the effect remains constant. Dose dependency revealed that this effect was maximal when sVpr1-96 was applied at a concentration of approximately 10 nM (data not shown). Parallel to the estimation of the RT activities released, the number of live cells was measured in the infection experiments by using a trypan blue exclusion method. This estimation was conducted each time of medium exchange and the numbers of cells were plotted as function of time (Figure 3B). Those results clearly show that, compared to the control culture, the number of living cells is approximately 1.5 fold increased in the presence of sVpr1-96. In general, and also as seen in the control culture (Figure 3B), the spread of infection in HIV-1 infected culture is characterized by a maximum of cytopathic effect at the time of maximal virus replication followed by a drastic decline in the number of living cells. This situation is different in the presence of sVpr1-96, the number of living cell starts

to decline with lower kinetic as in the control culture at day nine post infection. This phenomenon can be explained either by suppression of cytopathic mechanisms like HIV-1 induced apoptosis and/or cell fusion and syncytia formation. Alternatively, sVpr1-96 could merely increase the number of HIV-1 infected cells in the culture. Worth mentioning is the observation that this effect is not restricted to the replication of wild type virus and it also occurred with comparable intensity in PBMCs isolated from different donors. For this purpose, PHA/IL2 stimulated PBL derived from a different donor were infected with T cell tropic wild type virus HIV-1NL4-3 (Figure 3C) or with the chimeric macrophage-tropic virus NL4-3(AD8) (Figure 3D) and treated with 10 nM of sVpr1-96 or the control peptide Vpu32-81. Under the same conditions PBMCs were also infected with the vpu-deficient virus NL(AD8)-UDEL1 (Figure 3E) as well as with the vpr-deficient virus NL(AD8)deltaR (Figure 3F). It is well known that both, Vpr and Vpu can stimulate virus replication in PBMCs. In both situations and similar to the findings in cultures infected with the wild type virus HIV-1NL4-3 approximately a three to five fold increase in virus replication was observed in the presence of sVpr1-96.

In summary, sVpr1-96 stimulates virus replication in primary human T lymphocytes, and at the same time it causes an increase in the number of living cells. The latter effect occurs independently of the endogenous expression of the viral accessory proteins Vpr or Vpu.

Example 18:

sVpr1-96 complements the replication of vpr-deficient HIV-1-mutants in cultures of primary human monocytes/macrophages.

In order to test the impact of sVpr1-96 on virus replication primary human monocytes/macrophages "monocyte derived macrophages" (MDM) cells were isolated from blood lymphocytes of three HIV-seronegative healthy individuals (Donors #1 to 3). Cells were differentiated during a 14 day incubation period. Parallel cultures of MDM isolates were infected with equal infectious doses of chimeric macrophage-tropic virus NL(AD8) as well as the isogenic vpr-deficient mutant virus NL(AD8)deltaR. With the start of the infection, parallel cultures were treated with 10 nM sVpr1-96 as well as with 10 nM of the control peptide Vpu32-81. 90% of the culture medium was replaced with fresh medium and peptide solutions every three days. Aliquots of the cell culture supernatants were harvested during a two-month cultivation period and frozen at -80°C.

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Finally, the amount of virus associated RT activity was estimated and plotted as a function of time, the corresponding replication profiles are demonstrated in Figure 4. While wild type virus NL4-3(AD8) was able to establish a productive infection in MDM culture with maximum of virus replication on day 24 post infection the replication and spread of infection in cultures infected with the vpr-deficient mutant NL(AD8)deltaR was significantly reduced (Figure 4A). In MDM cultures derived from donors #1 and #2 replication of the vpr-deficient mutant NL(AD8)deltaR was approximately only 15% of the replication of the wild type virus (Figure 4B,C). In the MDM culture derived from donor #3 for NL(AD8)deltaR even no productive infection at all was detectable (Figure 4D). The reduced replication vpr-deficient mutant NL(AD8)deltaR is consistent with the previously described function of the accessory function of Vpr in macrophages/monocytes. However, continuous addition of 10 nM sVpr1-96 to the cell culture medium during the entire course of infection did not positively affect the replication of the wild type virus NL4-3(AD8), the replication profile was even reduced to approximately ~60% of the non treated culture (Figure 4A). In contrast, addition of 10 nM sVpr1-96 stimulated significantly replication of the vpr-deficient mutant NL(AD8)deltaR: the virus production in MDM cultures derived from 3 different donors was enhanced by the presence of sVpr1-96 in the culture medium. The addition of exogenous peptide sVpr1-96 thus rescued virus replication of vpr-deficient viruses to near wild type levels as demonstrated by the replication profiles in the presence of the peptide (Figure 4C, D). Even in MDM cultures that had no detectable spread of infection for the vpr-deficient mutant NL(AD8)deltaR, addition of exogenous peptide sVpr1-96 completely restored virus replication to wild type levels (Figure 4D).

In summary, while exogenous peptide sVpr1-96 has no positive effect on the replication of wild type virus in cultured human monocytes/macrophages, it can complement the accessory function of endogenous Vpr in vpr-deficient virus mutants and thus restore virus replication competence of those mutants to near wild type levels.

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Example 19:

¹H NMR-Spectroscopy on sVpr1-96

First NMR spectra of sVpr1-96 -were recorded in 2mM peptide solution (17mg/mL) in 50% (w/v) tri fluoro ethanol (TFE). The 1D spectra (shown as abscissa and ordinate in Figure 5) demonstrates relatively strong line broadening that is quite unusual for this small 96 amino acid protein, providing evidence that, under the solution conditions, the peptide sVpr1-96 at least partially may have the tendency to undergo self-association. The peptide was also dissolved in aqueous systems without any salt or buffer, the addition of dithiothriethiol (DTT) had no detectable effect on the spectra, thus excluding the formation of disulfide bounds. The NMR data, however, do not provide clear evidence for the existence of high order oligomeric structures above 100 kDa, as demonstrated previously using different techniques (Zhao et al., 1994). In case such oligomeric structures (presumably in the order of hexamers) of sVpr1-96 would exist, such complexes would tumble very slowly and cause broad signal in the spectra. However, such a phenomenon was not detectable in the spectra recorded for sVpr1-96. Individual spin systems could be identified, for instance three times alanine and one time for valine. As those spin systems are distributed throughout the entire molecule, this observation is also inconsistent with the existence of the peptide as a hexamer. Those preliminary data are rather indicative for the presence of sVpr1-96 in a steady state between monomeric and dimeric structures, both of which tend to induce signal broadening.

In order to further solve the problem of line broadening, 2D TOCSY and NOESY spectra sVpr1-96 were recorded that altogether brought evidence for the presence of cis/trans isomeric form in the molecule: certain parts of sVpr1-96 exhibit extra intensive broad signals while sharp cross peaks were identified for another regions of the molecule in the 2D spectra. In the lower field of the TOCSY spectra (10-9.3 ppm), three signals corresponding to tryptophan side chains were identified (Figure 5A). Further enlargement of those cross peaks provided additional insight into the heterogeneity of those signals; beside the main signal there are at least two minor signals. The same observations were made for histidine (Figure 6B) and arginine (Figure 6C). These types of amino acid side chains are distributed over the entire length of the molecule sVpr1-96 (tryptophan in positions 18, 38 and 54; histidine in positions 33, 40, 45, 71 and 78, and arginine in positions 12, 32, 36, 62, 73, 77, 80, 87, 88 and 90). Furthermore, those cross peaks represent very sharp signals. Consequently, it could be assumed that the observed phenomenon, particularly the relatively broad

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lines in the 1D-spectra and the signal multiplication in 2D spectra is not the result of oligomerization as reported previously for Vpr (Zhao et al., 1994).

The individual enlargement of 2D spectra shown in figure 6 point to the relative existence of approximately 20% heterogeneity within the investigated signals that, all together, is typical for cis/trans isomerism caused presumably by the four proline residues in sVpr1-96. Such isomers represent an unusually high cis-content (up to 40%) of proline residues in positions 14 and 35, that could be attributed to the close vicinity of aromatic amino acid side chains to those proline residues.

In order to further investigate the so far first observation of cis/trans isomerism in sVpr1-96, the following short peptides were synthesized and analyzed according to the invention:

sVpr1-20

(1M - E - Q - A - P - E - D - Q - G - P10 - Q - R - E - P - Y - N - E - W - T - L20), and

sVpr21-40

(21E - L - L - E - E - L - K - S - E - A30 V - R - H - E - P - R - I - W - L - H40).

In the investigated 1D and 2D NMR spectra, multiple signals corresponding to different proline residues were identified for both peptides, sVpr1-20 sVpr21-40. For the peptide sVpr21-40, approximately 10% of the molecule was found in the cis conformation. Similar results were obtained for the peptide sVpr1-20 with three different cis proline structures accounting together up to 30% of the entire molecule being in cis conformation.

In summary, it was observed that cis/trans isomerism of proline residues are the major cause for the heterogeneous structures of the sVpr1-96 full length molecule. Based on the NMR calculation of the short peptides it can be predicted that for the entire molecule sVpr1-96 approximately 40% single cis, 6% double cis, and 0,4% tippel cis conformation of all proline residues may exist, while only 59% of all proline residues exist in trans conformation.

In a further step of the procedure, the proline residues are identified which contribute primarily to the observed heterogeneity. Those proline residues are exchanged in sVpr1-96 by asparagine, a

conservative amino acid exchange. The aim is to replace proline with amino acid side chains that have a similar impact on the folding of the protein backbone but do not participate in the cis/trans isomerism. The exchange of proline to asparagine is based on previously published studies on the structure correlation matrix between amino acid side chains and protein folding (Livingston and Barton, 1996). Such constructed Vpr mutants that cannot undergo cis/trans isomerism are the ideal substrate for structural analyses using NMR and x-ray crystallography. The biological activity of such proline to asparagine mutated Vpr molecules can also be established.

Example 20:

Materials and Methods.

Example 20a:

Molecular HIV-1 clones and plasmid construction.

For the construction of T cell tropic viruses based on the molecular clone HIV-1NL4-3, the previously published plasmid pNL4-3 was used (Adachi et al., 1986). For the construction of macrophage-tropic viruses, the previously published plasmid pNL4-3(AD8) was used (Schubert et al., 95; Freed et al., 1995; Freed and Martin, 1994). This plasmid encodes the molecular infectious DNA of a macrophage-tropic virus that carries the env gene of the primary macrophage-tropic virus AD8 (Theodore et al., 1995) inserted into the back bone T cell tropic viruses HIV-1NL4-3 (Freed and Martin, 1994). For the replication competence of HIV-1 in monocytes and macrophages, a domain of the env gene is necessary, including the V3 loop (Schubert et al., 95; Freed et al., 1995; Freed and Martin, 1994; O'Brien et al., 1990; Shioda et al., 1991). For the construction of vpr-deficient mutant pNL(AD8)-deltaR, the plasmid pNL4-3(AD8) was linearized with the restriction enzyme EcoRI at base pair position 5743, the overhanging ends were filled in with DNA-polymerase I and the plasmid was then religated. This procedure causes a frame shift mutation within the vpr reading frame resulting in the expression of a N-terminal fragment of Vpr, which is unable to perform any of the so far described biological activities of Vpr.

Example 20b:

Cell culture.

Hela cells were cultivated in Dulbecco's' modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. PBMCs ("peripheral blood mononuclear cells") were isolated by gradient

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centrifugation of blood lymphocytes of healthy HIV-1-seronegative individuals, and cells were aliquoted and frozen in liquid nitrogen. Two days prior to infection, PBMC cultures were stimulated with phytohaemagglutinin (PHA, 1mg/ml) and human interleukin-2 (hIL-2, 20 U/ml). The treatment with hIL-2 was continued throughout the entire course of the experiment. MDM ("monocyte-derived macrophages") were isolated by counter current elutriation using standard and previously published methods (Schubert et al., 1995; Ehrenreich et al., 1993). Adherent cultures of MDM were pre-cultured in DMEM supplemented with glucose (4.5 g/L), penicillin (50 U/ml), streptomycin (50 mg/ml), L-glutamine (2mM), sodium pyruvate (1mM), and 10% human serum using a modified method described by Lazdins et al. (1990). Following a two week period of differentiation, MDM were resuspended, harvested, and re-plated at a concentration 0.5×10^6 cell per ml, and further incubated for approximately another two to three days.

As a control for potential contamination with CD4+ T lymphocytes -parallel cultures of MDM from each were incubated with the T cell tropic virus HIV-1NL4-3. In all experiments, no infection HIV-1NL4-3 could be established in any of the MDM cultures used in those experiments attesting for the absence of CD4+ T lymphocytes in our MDM preparation.

Example 21:

Transfection and generation of virus stocks.

For the purpose of generating virus stocks, plasmid DNA of molecular HIV-1 clones were transfected in HeLa cells using calcium phosphate precipitation. Confluent cultures of HeLa cells (5×10^6 cells) were transfected with 25 µg plasmid DNA associated with calcium phosphate crystals produced according to the method described previously by Graham and van der Eb (1973). Cells were incubated and subsequently subjected to a glycerol shock according to a method described previously by Gorman et al. (1982). For production of concentrated virus stocks, cell culture supernatant of the transfected cultures were harvested two days after transfection. Cells and debris were separated by centrifugation ($1,000 \times g$, 5 min, 4°C) and filtration (0.45 µm pore size). Virus particles were pelleted by ultra centrifugation (Beckman SW55 Rotor, 1.5 hr, 35,000 rpm, 10°C) and resuspended afterwards in 1 ml of DMEM Medium. Virus stocks were sterilized by filtration (0.45 µm pore size), aliquoted, and frozen at -80°C. Individual virus stock standardized by estimation of

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the amount of RT (reverse transcriptase) activity using a previously described assay (Willey et al, 1988) based on the incorporation of [32P]-TTP into an oligo(dT)-poly(A) template.

Example 22:

¹H NMR on sVpr peptides

1D and 2D ¹H NMR spectra were recorded on a DMX 600 Bruker NMR-Spectrometer without spinning at 300°K. Spectra were calibrated based on the proton in TFE at 3.95 ppm.

More details are given in the figure legend. --

IN THE CLAIMS

Please cancel claims 1-30 without prejudice to Applicants' right to present the subject matter of these claims in a subsequent amendment or application, and add new claims 31-33 as follows:

31. (NEW) A synthetic peptide comprising a regulatory virus protein R (Vpr) of the human immunodeficiency virus type 1 (HIV-1).

32. (NEW) The synthetic peptide of claim 1, comprising:

- Sub. 1
Sub. 2
- (a) a 96 amino acid Vpr protein (sVpr¹⁻⁹⁶);
 - (b) a 47 amino acid N-terminal peptide (sVpr¹⁻⁴⁷);
 - (c) a 49 amino acid long C-terminal peptide (sVpr⁴⁸⁻⁹⁶); or
 - (d) a fragment of at least approximately 15 amino acids of any one of (a)-(c).

33. (NEW) The synthetic peptide of claim 32, wherein the fragment comprises sVpr¹⁻²⁰ or sVpr²¹⁻⁴⁰.